Polysaccharide capsule composition of pneumococcal serotype 19A subtypes: Unaltered among subtypes and independent of the nutritional environment

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ABSTRACT

Serotype 19A strains have emerged as a cause of invasive pneumococcal disease after the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) and serotype 19A has now been included in the recent thirteen-valent vaccine (PCV13). Genetic analysis has revealed at least three different capsular serotype 19A subtypes and nutritional environment dependent variation of the 19A capsule structure has been reported. Pneumococcal vaccine effectiveness and serotyping accuracy might be impaired by structural differences in serotype 19A capsules. We therefore analyzed the distribution of 19A subtypes collected within a Swiss national surveillance program and determined capsule composition in different nutritional conditions with high-performance liquid chromatography (HPLC), gas chromatography – mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). After the introduction of PCV7 a significant relative increase of subtype 19A-II and decrease of 19A-I occurred. Chemical analyses showed no difference in the composition as well as the linkage of 19A subtype capsular saccharides grown in defined and undefined growth media being consistent with a trisaccharide repeat unit composed of rhamnose, N-acetyl-mannosamine and glucose. In summary, our study suggests that no structural variance dependent of the nutritional environment or the subtype exists. The serotype 19A subtype shift observed after the introduction of the PCV7 can therefore not be explained by selection of a capsule variant. However, capsule composition analysis of emerging 19A clones is recommended in cases where there is no other explanation for a selective advantage such as antibiotic resistance or loss or acquisition of other virulence factors

INTRODUCTION

The polysaccharide capsule is a major virulence factor of the human pathogen *Streptococcus pneumoniae* (pneumococcus) and more than 90 different capsular types are known today, which differ in the chemical structure of their capsular polysaccharides (1). These differences are reflected in the type-specific reaction with anticapsular antibodies, by which a serotype is determined and cross-reactive serotypes are pooled into serogroups. The serogroup 19 contains, among others, serotypes 19F and 19A, which belong to the clinically most relevant serotypes (2). Based on both genetic background and chemical analyses the serotype 19A and 19F oligosaccharide repeating unit structures have been determined to be trisaccharides of glucose (Glc), rhamnose (Rha) and N-acetyl-mannosamine (ManNAc), differing only in the glycosidic linkage between glucose and rhamnose:

19F: →2)-α-L-Rha-(1→PO_4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→

19A: →3)-α-L-Rha-(1→PO_4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→ (1, 3-5).

Based on chemical analyses two types of 19A oligosaccharide structures have been described (6, 7). In addition to the genetically proposed structure above, an alternative with a serotype 19F backbone and two side chains of β-D-GlcNAc-(1→3)-β-D-Gal- (1→P→2) and α-L-Fuc-(1→P→3) has been reported based on chemical analysis (7). The polysaccharide structures appear to vary with different *in vitro* growth conditions (6). Influence of the nutritional environment on the pneumococcal polysaccharide capsule could have biological consequences, as this would potentially impair any intervention or test targeting the pneumococcal capsule. For example, the fungus *Cryptococcus neoformans* is known to be able to change the capsule structure in vitro and also during infection (8-11), and those changes have been shown to lead to altered antigenicity (8, 11).

Because polysaccharides for pneumococcal vaccine production are derived from *in vitro* cultures, a nutrient-dependent variation could lead to antigenic preparations which differ from the *in vivo* antigen.

Capsule variation could also impair diagnostic procedures such as classical serotyping, which is based on polysaccharide specific antigen-antibody reaction.

Serotype 19A strains have emerged after the introduction of the seven-valent pneumococcal vaccine, and were subsequently included in the thirteen-valent vaccine now recommended in most countries (12, 13). The emergence of serotype 19A after the introduction of the seven-valent vaccine was surprising, as a cross-protection was expected due to close chemical similarity to the serotype 19F capsule as it was observed for serotypes 6A and 6B which also differ only by one glycosidic linkage (14, 15). Furthermore, 19F-19A cross-protection had been observed to a certain degree in an animal model (16). Recent work suggests a conformational difference in polysaccharide structure, which might explain the reduced cross-protection (17, 18).

At least three different 19A capsule subtypes are known based on the genetic arrangement of the capsule gene locus compared to a reference strain (19). In addition to various SNPs along the capsule operon, most characteristically, subtypes I and II have an inverted rmlD gene, which is the last gene in the rhamnose synthesis pathway (3). To our knowledge, no variations in the capsule structure have been described for different subtypes.

Given the recent discovery of 19A capsular subtypes and previous reports of structural variants as well as the introduction of serotype 19A in the 13- valent pneumococcal conjugate vaccine (PCV13) we aimed to determine the epidemiology and capsule composition of different 19A subtypes in different nutritional environments.

MATERIAL AND METHODS

Bacterial strains and serotype 19A subtype analysis. Serotype 19A subtype strains were selected from a Swiss national pneumococcal surveillance program (20). In order to detect the different described 19A subtypes (19) we analyzed 158 pneumococcal serotype 19A isolates derived from the upper respiratory tract of infant and adult outpatients with signs of upper respiratory tract infection (20). Antimicrobial susceptibility for penicillin, sulfamethoxazole / trimethoprim (SXT) and erythromycin was determined as previously described (20). In brief, minimum inhibitory concentration (MIC) for penicillin non-susceptibility was ≥ 0.06 mg/L while for erythromycin and SXT the disk diffusion method was performed (intermediate and resistant were considered as non-susceptible). A two-step PCR protocol was used to determine the 19A subtype of each strain using the following conditions. The first PCR was done with two primer pairs: rmlb_1_f -GAT GGT GAG AAG AAC AAT AAG; rmlb_2_f - GAC GGT GAG AAG AAC AAC AAG; rmld_1_r- CTT CAT TAC GTT CAT CCA ATA and rmld_2_r CAG CTG AAG ACA CCA CTT GGT. PCR conditions were initial heat activation 6 min 95°C, 30 cycles of 30s 95°C, 20s of 60°C and 90s of 72°C with a final extension of 5 min 72°C. The reaction mixture contained 2.5 µL of FastStart *Taq* reaction buffer without $MgCl_2$, 2.7 µL of 25 mM $MgCl_2$ stock solution, 4 µL of 1.25 mM deoxynucleoside triphosphates (0.2 mM final), 0.2 µL (1 U) of FastStart *Taq* polymerase (all from Roche Molecular Biochemicals, Rotkreuz, Switzerland), and 0.25 µL of each primer (100 µM stock resulting in 1 µM final concentration, Microsynth AG, Balgach, Switzerland) in a total volume of 25 µL. PCR products were visualized on 1% agarose gels. A resulting PCR product of 560 bp indicated subtypes 19A-I and 19A-II whereas a band of 425 bp indicated subtype 19A-III. For isolates within the first group (19A-I/19A-II), a second PCR reaction was performed to discriminate between the two groups using the following primers: wzg_2_f - AGT TGA TTC GTC CAT CCA CAC T; wzg_3_f -GGA ATT GAC ACA TAT GGT CCT and wzh_r -GCC AAG AGA GCC TTG CTT TCC. The resulting PCR products were 654 bp and 833 bp for types 19A-I and 19A-II, respectively.

Strains were further characterized with *plyNCR*-RFLP as previously described (21, 22). We selected the following strains for further analysis of the capsule composition (Table 1): 109.44 and 501.14 (subtype 19A-I), 501.24 (subtype 19A-II), and 412.49 (subtype 19A-III). For all selected subtype test strains, multi locus sequence typing (MLST) was determined as previously described (22). In addition, we included the internationally spread Hungary-19A-6 strain (23), which was classified as a 19A III. Serotype 19F strains 505.32 and B201.73 were selected for comparison as the polysaccharide repeat unit contains the same backbone monosaccharides but different glycosidic linkages between the repeat units. Both strains are clinical isolates derived from Swiss national surveillance programs (20, 24). In addition a capsule knockout mutant of strains B201.73 was generated as previously described (25, 26) to assess the amount of background signal in capsule extracts. Commercially available capsule polysaccharide of serotype 19A from the American Type Culture Collection (ATCC, Molsheim Cedex, France) was used as a reference standard.

Chi-Square and Fisher exact tests were used to calculate p values for epidemiological analyses. A value of $p \leq 0.05$ was considered significant. In addition, we used a multivariate logistic regression model to ascertain the strength of the association between PCV7 era and 19A subtypes, and adjusted for potential confounders like age (0-1 (base), 2-4, 5-15 and >15 years), sex (male gender as base), penicillin resistance (susceptible chosen as base), erythromycin resistance (susceptible chosen as base), SXT resistance (susceptible chosen as base) and geopraphical origin (east Switzerland as base). Adjusted odds ratios (aOR) with 95% confidence intervals (95%CI) were received (table S1). Trends over time in the prevalence of different 19A subtypes before the introduction of PCV7 were analyzed using linear regression.

Growth conditions, polysaccharide purification, hydrolysis and high performance liquid chromatography (HPLC). Strains were handled and grown as described previously (25). The undefined growth medium pneumococcal inoculation medium (PIM) which has been reported to alter 19A capsule composition (6), as well as a chemically defined medium

(CDM) by van den Rijn and Kessler (27) were used. CDM was supplemented with 5 mg/L choline chloride (28), but made without monosaccharides to allow modification of the type and concentration of the carbon source for each experiment. Monosaccharides were added to the CDM and the mixture was sterile filtrated using a 0.22 µm filter unit (TPP, Trasadingen, Switzerland). Two forms of CDM were created, one supplemented with 55 mM Glucose and the other one mimicking the composition of the salivary mucin MG1, i.e.: N-aceteylneuraminic acid (NeuNAc):fucose (Fuc):galactose (Gal):N-acetylglucosamine (GlcNAc):N-acetylgalactosamine (GalNAc) = 1:5:4:3:1 (29)). For one experiment pooled human saliva from ten healthy volunteers was collected using Salivette (Sarstedt, Nümbrecht, Germany). After centrifugation with 1500 x g for 2 minutes samples were pooled and sterile filtrated with 0.22µm centrifugal filter units (Millipore, Billerica, MA) at 5000 x G for 20 minutes as previously described (30).

Capsules of strains grown in different growth media were released by overnight incubation in 1% phenol, separated from the bacteria by centrifugation and filtration, and then purified with sodium acetate/ethanol precipitation, followed by protease and nuclease digestion of remaining contaminants, and finally cut-off filtrated as described (25). The extracted polysaccharides were then completely hydrolyzed by trifluoroacetic acid (TFA) (31). The monosaccharide composition of capsular polysaccharides of strains grown in CDM was determined by high performance liquid chromatography (HPLC) analysis of fluorescently labelled monosaccharides as previously described (25, 32-35), whereas the PIM extracts were analyzed on a system consisting of an ASI-100 autosampler and P680 HPLC pump (DIONEX, Sunnyvale, CA, USA) with an injection volume of 20 µL per sample. Separation of the monosaccharide was done with a flow rate of 0.85 mL/min as follows: 6% solvent B isocratic for 35 minutes followed by a linear gradient from 6 to 12% solvent B over 20 minutes. Then, the column was washed with 100% solvent B for 10 minutes and 100% solvent A for 15 minutes followed before re-equilibrating the system with 6% solvent B for 10 minutes. Total run time was 90 minutes and data was collected for 55 minutes using an L-7480 Fluorescence Detector (Merck Hitachi, Darmstadt, Germany). A Luna 5 mm, C18

column (Phenomenex, Torrance, CA) was used for separation and column temperature was maintained at 24 °C using a TCC-100 column oven (Thermo Scientific Dionex, Reinach, Switzerland). Peaks were identified by comparing retention time with monosaccharide standards analyzed in the same run (Figure S1A). As N-acetylated aminosugars are deacetylated during hydrolysis with TFA (36), deacetylated aminosugars were used as standards for their acetylated counterpart (e.g., mannosamine for N-acetyl-mannosamine). Negative controls included in each experiment were medium negative control for growth, extraction, hydrolysis and HPLC. HPLC raw data was exported into GraphPad Prism (Version 5, GraphPad Software, Inc.) for creating figures.

Gas chromatography – mass spectrometry (GC-MS). GC-MS analysis of alditol acetates of the polysaccharide hydrolysates from strains 109.44 grown in PIM and 501.24 grown in PIM, pooled saliva, and CDM glucose was performed as a control, as previously described (37, 38).

Nuclear magnetic resonance spectroscopy (NMR). NMR data were collected on a Bruker Avance II (500 MHz; ^{1}H) spectrometer equipped with a 1.7 mm triple-resonance (^{1}H, ^{13}C, ^{31}P) microprobe head. The samples were extracted as described above and prepared as follows: The full amount of each capsule extract (~4 – 5 mg) was dissolved in 50 µl of D_2O and 40 µl of the resulting mixtures were transferred into 1.7 mm NMR tubes. The water resonance was suppressed using a classical presaturation scheme. HSQC spectra were collected on a Bruker Avance III HD (600 MHz) spectrometer equipped with an inverse 5mm TCI helium cryoprobe. All spectra were acquired at a regulated temperature of 298 K and calibrated to the residual water peak (4.766 ppm). For the interpretation of the received carbon and hydrogen shifts, results from previous studies were used as guidance for the capsule structure determination of the 19A capsule extracts (4, 39, 40).

RESULTS

Epidemiology of non-invasive 19A subtypes. We first aimed at analyzing the distribution of the different pneumococcal serotype 19A subtypes within the Swiss Sentinel Network collection of upper respiratory tract isolates. PCV7 had been recommended in Switzerland since late 2005 to all children under the age of 24 months in a three dose schedule given at 2, 4 and 12 months and since August 2006 the vaccine has been fully reimbursed by the mandatory Swiss health insurance. Serotype 19A strains isolated from the upper respiratory tract between 1998 and 2011 were analyzed. In total, 158 19A isolates were screened for the three described subtypes using a two-step PCR protocol described above. Between 1998 and 2005, we identified 30 (57.7%), 5 (9.6%) and 17 (32.7%) isolates belonging to subgroups 19A-I, 19A-II, and 19A-III, respectively. After 2006 until 2011, a significant relative difference was noted overall (p=0.02) but also individually for the subgroups 19A-I (p=0.02) and 19A-II (p=0.02) as 40 (37.7%), 28 (26.4%) and 38 (35.8%) isolates were detected with the 19A-I, 19A-II, and 19A-III subtypes, respectively (Figure 1A). There was still a significant shift even after data from the PCV7 introduction year (2006) were excluded (p=0.03). We found no evidence for a difference of 19A subtypes distribution according to age (Pre versus post introduction of PC7 eras over age groups of ≤1, 2-4, 5-15 and >15 years; Fisher's exact test; p= 0.3). In addition, there was no indication of a time trend for the frequency of 19A subtypes before the PCV7 was present (Data not shown). As for molecular types derived by *plyNCR*-RFLP typing, *plyNCR*-RLPF types 1 (55.7%) and 16 (18.4%) were the most frequent within the 158 strains but we did not detect a significant shift of molecular types between pre and post PCV7 introduction era (Figure 1B). In addition we revealed that antibiotic resistances within the 19A strains were generally high and that 19A II is significantly more susceptible towards penicillin, erythromycin, and SXT as compared to the other subtypes (Figure 1C). However, calculating a multivariate logistic regression model we confirmed that there was strong evidence that, compared to 19A I, the odds of observing subtype 19A II after introduction of PCV7 was 6 times higher than before PCV7, and that this

association was independent of antimicrobial resistance, geographical region, age or sex (p = 0.005; **Table S1**).

We then selected at least one strain of each subtype for further laboratory analysis to determine capsular composition of the different subtypes (Table 1). Selected subtype strains represented different genetic backgrounds as indicated by MLST and *plyNCR*-RFLP analysis (Table 1).

Method validation. To assess the degree of contamination of capsule extracts with cell wall and other components, we first compared the monosaccharide composition of hydrolyzed capsule extracts of a serotype 19F clinical isolate B201.73 with extracts of its isogenic capsule knockout mutant (B201.73Δcps) by HPLC with fluorescence detection (Figure S1B). No rhamnose peaks were detected for the strain without a capsule, but small amounts of glucose and higher amounts of amino sugars including mannosamine could be detected, most likely deriving from cell wall polysaccharide (CWPS) and from murein layer. Furthermore, hydrolysis with TFA is known to produce varying amounts of hydrolysis-derived (di)saccharides (Figure S1B). This contamination was usually present in capsule extracts from tested strains and was higher in amount as compared to the commercially available purified pneumococcal polysaccharide (Figure 2). Furthermore, after complete hydrolysis a linkage analysis is not possible and only monosaccharide determination can be done (e.g., same chromatogram for serotype 19A and 19F). Therefore, we used the HPLC method only as a screening tool for additional neutral monosaccharides under different growth conditions (i.e. galactose and fucose).

Capsule composition analysis of 19A subtypes by HPLC and GC-MS. We then analyzed the capsule composition of a strain for each subgroup (109.44 and 501.14 (subtype 19A-I), 501.24 (subtype 19A-II), and Hungary-19A-6 and 412.49 (subtype 19A-III) grown in CDM supplemented with 55 mM glucose. We did not detect additional neutral monosaccharides and comparison of the used isolates revealed no differences among the strains indicating

the same monosaccharide backbone built of glucose, rhamnose and (N-acetyl)-mannosamine. As expected, HPLC chromatograms were not able to differentiate 19A from 19F as both serotypes contain the same monosaccharide compositions (Figure 2A). We then analyzed the capsular extracts from the ATCC standard (Figure 2B) and from 19A subtypes grown in the undefined medium PIM (Figure 2C). Again, no additional neutral monosaccharides were detected and thus reported additional monosaccharides (galactose and fucose would have been expected on the neutral side based on the literature (6)) were not seen in our preparations. As HPLC allows only the identification of peaks based on their retention time in comparison to standards, the presence of the neutral monosaccharides in capsule extracts was confirmed for 109.44 (19A-I) grown in CDM with 55 mM Glucose (Figure S3A) and PIM (Figure S3B) and 501.24 (19A-II) grown in PIM (Figure S3C) with GC-MS (which revealed glucose and rhamnose in all preparations). GC-MS also confirmed a lower degree of contamination with cell wall and other compounds in the ATCC standard compared to subtype capsule extracts (Figure S3D).

To mimic saccharide nutrients present in the natural human environment of *S. pneumoniae*, 109.44 (19A-I) was grown in CDM with monosaccharides contained in human mucin (5.5 mM total concentration of the mucin building monosaccharides in ratios as determined for the salivary mucin MG1) and 501.24 (19A-II) was also grown in pooled human saliva collected from 10 healthy volunteers after sterile filtration. No additional neutral monosaccharides were identified to be present in the polysaccharide capsule extracted from the strains grown under these conditions compared to capsule extracted from strains grown in CDM supplemented with glucose or PIM but the signal to noise ratio was much lower in saliva grown capsule (Figure S2 and S3E).

NMR analyses reveal no structural difference between subtypes.

In order to confirm the proposed structures of the oligosaccharides we compared the anomeric region (4.8-6.0 ppm) in the ^{1}H-NMR of all strains grown in CDM supplemented with 55mM glucose (Figure 3A). We were able to clearly differentiate the capsule of serotype 19A

strains from 19F capsule. Comparison of carbon and hydrogen shifts with previously reported NMR spectra confirmed the following structures for serotype 19F: →2)-α-L-Rha-(1→PO4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→ and for serotype 19A: →3)-α-L-Rha-(1→PO4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→ . In addition, no differences among the serotypes 19A subtypes were detected. We then performed an identical ^{1}H-NMR analysis of all strains grown in PIM which has been reported to alter the composition of 19A capsule (6). The spectra were again consistent within 19A subtypes but different from serotype 19F capsular polysaccharide (Figure 3A). Based on those results, it can be stated that the capsule composition is independent of tested growth medium as the patterns were identical to each other and to the ATCC reference 19A polysaccharide. Only a slight shift was observed for the capsule analysis of the Hungary 19A-6 strain. However, the overlay of the 2D ^{1}H-^{13}C HSQC-NMR spectra of this isolate grown in PIM and CDM supplemented with glucose revealed identical patterns for the H-atoms of the anomeric region as illustrated for rhamnose, N-acetyl mannosamine and glucose (Figure 3B). The full 2D Spectrum is shown in Figure S4.

DISCUSSION

In this study we demonstrated that the distribution of pneumococcal serotype 19A subtypes changed after the implementation of PCV7 in Switzerland. However, all analyzed capsular extracts from serotype 19A subtypes grown in defined medium CDM and the undefined medium PIM showed a capsule composition which was consistent with the one proposed based on genetic analysis. Furthermore, we did not find any evidence for additional side chains altered repeat units or linkage changes under different nutritional conditions. .

During the time of widespread use of PCV7 in Switzerland the serotype 19A subtype distribution changed substantially compared to the pre-vaccine period. Similar shifts with decreasing 19A-I and increasing 19A-II subtypes after the introduction of PCV7 have also been reported in the Netherlands (19). Furthermore, although classified as group III by PCR, based on its genome sequence the Hungary 19A-6 strain contains an additional insertion element downstream of the last cps gene (*rmlD*) and differs from the subgroup III strains as defined by Elberse et al. (19) which could suggest an additional serotype 19A subgroup.

Given the identical capsule structure for all tested 19A subtypes as revealed for the first time in our study using NMR, HPLC and GC-MS, no alteration of vaccine effectiveness due to capsular differences between individual subtypes is suggested. Furthermore, the lack of anti-19F antibody cross-protection against serotype 19A can therefore not be explained by selection of 19A subtypes with altered capsule structures which supports recent discoveries suggesting a general low protection due to conformational difference in polysaccharide structures of 19F and 19A (17, 18).

However, other factors could explain the observed subtype shift after the introduction of PCV7 in Switzerland. For example, changes in 19A subtypes could be due to changes in clonal distribution although we did not detect a significant shift of molecular types as determined by *plyNCR*-RFLP. However, additional typing methods or whole genome

sequencing might be more appropriate to investigate this hypothesis. As for antibiotic resistance being a potential driver for the subtype shifts, we revealed that 19A II is significantly more susceptible to antibiotics as compared to the other subtypes indeed, but as 19A II increases in the PCV7 era, increasing antibiotic resistance can therefore not explain the changes in the distribution, though antibiotic resistance within serotype 19A strains is generally high as previously shown (41). A subtype redistribution under increasing vaccine selection pressure after the introduction of PCV13 would be unexpected, although it has been speculated that there might be a difference in capsule thickness for different subtypes which might lead to differences in opsonophagocytotic susceptibility (19).

Previously reported experiments showing additional side chains in 19A isolates grown in PIM could not be replicated (6). An explanation for this finding might be a strain-specific genetic alteration in previous studies. Indeed, it has been recently reported that serotype 11D has two different capsular polysaccharide repeating units in a ratio of 1:3 (25% and 75%, respectively) due to a bispecific transferase WcrL (42). Although precursors of capsular galactose and N-acetyl-glucosamine might be available due to their synthesis in other pathways (43), the (environment-dependent) addition of 2 side chains comprised of three additional monosaccharides is expected to be reflected with at least additional glycosyltransferase enzymes within the capsule locus involved in their linkage, and a switch of the glycosidic linkage between rhamnose and glucose would suggest mutations resulting in bispecificity or two copies of the wzy polymerase (3). To our knowledge, neither of these two possibilities have been detected in 19A strains (3). However, variation of the repeat unit structure would be suspected to have an influence on the antigenicity thus resulting in suspect serotyping results (i.e., less reactive with anti-19A antiserum) which were not observed in this study. However, given the increasing vaccine selection pressure after the introduction of PCV13, we recommend capsule structure determination of emerging clones and/or serotypes given recent discoveries of novel serotypes and capsule variants (42, 44-47). The importance of chemical capsule structure analysis is also highlighted by a recent

analysis of serotype “6E” capsule, demonstrating that this potential new serotype determined at the genetic level produces capsular polysaccharide identical to 6B capsular polysaccharide (48).

A particular strength of this study is the use of multiple techniques for capsule structure determination, which enhances the reliability of reported findings. We first used an HPLC based approach to determine if additional neutral sugars were incorporated into the capsule and validated the methodology with GC-MS. As this technique requires complete hydrolysis a linkage analysis was not possible in this first step. Furthermore, although this method has been demonstrated to have high normalized recoveries of neutral monosaccharides rhamnose and glucose of serotype 19F monosaccharides (32), TFA hydrolysis has also been reported to be less satisfactory for polysaccharides with aminosugar moieties (32) and it has been reported that disaccharides can be formed during hydrolysis based on different stability of intramolecular bonds (32) which also occurred in our experiments. Results showed traces of contamination of cell-wall components in our preparations, which is a common co-extracted compound in pneumococcal capsule preparations probably because the capsule is covalently linked to the cell wall (32, 49, 50). To determine linkage analysis and further characterization of the capsule oligosaccharide repeat units, we performed additional 1D and 2D NMR analysis of polysaccharides from unhydrolyzed capsule extracts. The major limitation of this study is that the number of different 19A subtype strains analyzed was rather small. However, we chose at least one sample of each of the currently known subtypes and it can therefore be expected that our study is representative. Although we did not find evidence for nutrition-dependent variations of capsule structures, this cannot be considered as final proof for an absence of such variations. Furthermore, we did not have the same strains or capsule extracts for which a nutrient-dependent variation has been reported as a reference for the assays used (6).

In summary, the polysaccharide capsule composition for tested serotype 19A subtypes was consistently composed of the same trisaccharide repeat unit. Although we therefore do not

expect a structural advantage for certain subtypes it remains to be determined how the 19A subtype distribution will be affected by PCV13. We did not detect any nutritional environment-dependent alterations of the capsule composition. However, given the genetic plasticity of *S. pneumoniae* and current vaccine selection pressure, we propose to test the capsule composition of emerging serotype 19A clones, especially in cases where there is no other explanation for a selective advantage such as antibiotic resistance or loss or acquisition of other virulence factors.

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REFERENCES

1. **Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, Donohoe K, Harris D, Murphy L, Quail MA, Samuel G, Skovsted IC, Kaltoft MS, Barrell B, Reeves PR, Parkhill J, Spratt BG.** 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet **2:**e31.
2. **Hausdorff WP, Bryant J, Paradiso PR, Siber GR.** 2000. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis **30:**100-121.
3. **Morona JK, Morona R, Paton JC.** 1999. Comparative genetics of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* types belonging to serogroup 19. J Bacteriol **181:**5355-5364.
4. **Katzenellenbogen E, Jennings HJ.** 1983. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type 19A (57). Carbohydr Res **124:**235-245.
5. **Kamerling JP.** 2000. Pneumococcal polysaccharides: a chemical view, p81-114. *In* Tomasz A (ed), *Streptococcus pneumoniae*: molecular biology & mechanisms of disease. Mary Ann Liebert, Inc., Larchmont, NY.
6. **Lee CJ, Fraser BA, Boykins RA, Li JP.** 1987. Effect of culture conditions on the structure of *Streptococcus pneumoniae* type 19A(57) capsular polysaccharide. Infect Immun **55:**1819-1823.
7. **Lee CJ, Fraser BA.** 1980. The structures of the cross-reactive types 19 (19F) and 57 (19A) pneumococcal capsular polysaccharides. J Biol Chem **255:**6847-6853.
8. **Zaragoza O, Rodrigues ML, De Jesus M, Frases S, Dadachova E, Casadevall A.** 2009. The capsule of the fungal pathogen *Cryptococcus neoformans*. Adv Appl Microbiol **68:**133-216.
9. **Garcia-Hermoso D, Dromer F, Janbon G.** 2004. *Cryptococcus neoformans* capsule structure evolution *in vitro* and during murine infection. Infect Immun **72:**3359-3365.

10. **Cherniak R, Morris LC, Belay T, Spitzer ED, Casadevall A.** 1995. Variation in the structure of glucuronoxylomannan in isolates from patients with recurrent cryptococcal meningitis. Infect Immun **63:**1899-1905.

11. **McFadden DC, Fries BC, Wang F, Casadevall A.** 2007. Capsule structural heterogeneity and antigenic variation in *Cryptococcus neoformans*. Eukaryot Cell **6:**1464-1473.

12. **Tan TQ.** 2012. Pediatric invasive pneumococcal disease in the United States in the era of pneumococcal conjugate vaccines. Clin Microbiol Rev **25:**409-419.

13. **Gervaix A, Ansaldi F, Brito-Avo A, Azzari C, Knuf M, Martinon-Torres F, Tuerlinckx D, Tin Htar MT, Syrogiannopoulos GA.** 2014. Pneumococcal vaccination in Europe: schedule adherence. Clin Ther **36:**802-812 e801.

14. **Whitney CG, Pilishvili T, Farley MM, Schaffner W, Craig AS, Lynfield R, Nyquist AC, Gershman KA, Vazquez M, Bennett NM, Reingold A, Thomas A, Glode MP, Zell ER, Jorgensen JH, Beall B, Schuchat A.** 2006. Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet **368:**1495-1502.

15. **Vakevainen M, Eklund C, Eskola J, Kayhty H.** 2001. Cross-reactivity of antibodies to type 6B and 6A polysaccharides of *Streptococcus pneumoniae*, evoked by pneumococcal conjugate vaccines, in infants. J Infect Dis **184:**789-793.

16. **Jakobsen H, Sigurdsson VD, Sigurdardottir S, Schulz D, Jonsdottir I.** 2003. Pneumococcal serotype 19F conjugate vaccine induces cross-protective immunity to serotype 19A in a murine pneumococcal pneumonia model. Infect Immun **71:**2956-2959.

17. **Kuttel M, Gordon M, Ravenscroft N.** 2014. Comparative simulation of pneumococcal serogroup 19 polysaccharide repeating units with two carbohydrate force fields. Carbohydr Res **390:**20-27.

18. **Kuttel MM, Jackson GE, Mafata M, Ravenscroft N.** 2015. Capsular polysaccharide conformations in pneumococcal serotypes 19F and 19A. Carbohydr Res **406:**27-33.

19. **Elberse K, Witteveen S, van der Heide H, van de Pol I, Schot C, van der Ende A, Berbers G, Schouls L.** 2011. Sequence diversity within the capsular genes of *Streptococcus pneumoniae* serogroup 6 and 19. PLoS One **6:**e25018.

20. **Muhlemann K, Matter HC, Tauber MG, Bodmer T, Sentinel Working G.** 2003. Nationwide surveillance of nasopharyngeal *Streptococcus pneumoniae* isolates from children with respiratory infection, Switzerland, 1998-1999. J Infect Dis **187:**589-596.

21. **Hathaway LJ, Brugger S, Martynova A, Aebi S, Muhlemann K.** 2007. Use of the Agilent 2100 bioanalyzer for rapid and reproducible molecular typing of *Streptococcus pneumoniae*. J Clin Microbiol **45:**803-809.

22. **Zhou J, Enright MC, Spratt BG.** 2000. Identification of the major Spanish clones of penicillin-resistant pneumococci via the Internet using multilocus sequence typing. J Clin Microbiol **38:**977-986.

23. **McGee L, McDougal L, Zhou J, Spratt BG, Tenover FC, George R, Hakenbeck R, Hryniewicz W, Lefevre JC, Tomasz A, Klugman KP.** 2001. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the pneumococcal molecular epidemiology network. J Clin Microbiol **39:**2565-2571.

24. **Meichtry J, Born R, Kuffer M, Zwahlen M, Albrich WC, Brugger SD, Muhlemann K, Hilty M.** 2014. Serotype epidemiology of invasive pneumococcal disease in Swiss adults: a nationwide population-based study. Vaccine **32:**5185-5191.

25. **Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, Hauser C, Graber WA, Gore S, Kadioglu A, Muhlemann K.** 2012. Capsule type of *Streptococcus pneumoniae* determines growth phenotype. PLoS Pathog **8:**e1002574.

26. **Trzcinski K, Thompson CM, Lipsitch M.** 2003. Construction of otherwise isogenic serotype 6B, 7F, 14, and 19F capsular variants of *Streptococcus pneumoniae* strain TIGR4. Appl Environ Microbiol **69:**7364-7370.

27. **van de Rijn I, Kessler RE.** 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect Immun **27:**444-448.

28. **Desai BV, Reiter H, Morrison DA.** 2003. Choline starvation induces the gene licD2 in *Streptococcus pneumoniae*. J Bacteriol **185:**371-373.

29. **Thomsson KA, Prakobphol A, Leffler H, Reddy MS, Levine MJ, Fisher SJ, Hansson GC.** 2002. The salivary mucin MG1 (MUC5B) carries a repertoire of unique oligosaccharides that is large and diverse. Glycobiology **12:**1-14.

30. **Shelburne SA, 3rd, Sumby P, Sitkiewicz I, Granville C, DeLeo FR, Musser JM.** 2005. Central role of a bacterial two-component gene regulatory system of previously unknown function in pathogen persistence in human saliva. Proc Natl Acad Sci U S A **102:**16037-16042.

31. **Albersheim P.** 1968. A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. Carbohydr Res **5:**340-345.

32. **Talaga P, Vialle S, Moreau M.** 2002. Development of a high-performance anion-exchange chromatography with pulsed-amperometric detection based quantification assay for pneumococcal polysaccharides and conjugates. Vaccine **20:**2474-2484.

33. **Anumula KR.** 1994. Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection. Anal Biochem **220:**275-283.

34. **Anumula KR, Dhume ST.** 1998. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. Glycobiology **8:**685-694.

35. **Saddic GN, Dhume ST, Anumula KR.** 2008. Carbohydrate composition analysis of glycoproteins by HPLC using highly fluorescent anthranilic acid (AA) tag. Methods Mol Biol **446:**215-229.

36. **Kwon H, Kim J.** 1993. Determination of monosaccharides in glycoproteins by reverse-phase high-performance liquid chromatography. Anal Biochem **215:**243-252.

37. **Lehr T, Geyer H, Maass K, Doenhoff MJ, Geyer R.** 2007. Structural characterization of N-glycans from the freshwater snail *Biomphalaria glabrata* cross-reacting with *Schistosoma mansoni* glycoconjugates. Glycobiology **17:**82-103.

38. **Geyer R, Geyer H, Kuhnhardt S, Mink W, Stirm S.** 1982. Capillary gas chromatography of methylhexitol acetates obtained upon methylation of N-glycosidically linked glycoprotein oligosaccharides. Anal Biochem **121:**263-274.

39. **Abeygunawardana C, Williams TC, Sumner JS, Hennessey JP, Jr.** 2000. Development and validation of an NMR-based identity assay for bacterial polysaccharides. Anal Biochem **279:**226-240.

40. **Jennings HJ, Rosell KG, Carlo DJ.** 1980. Structural Determination of the Capsular Polysaccharide of *Streptococcus pneumoniae* Type-19 (F-19). Canadian Journal of Chemistry-Revue Canadienne De Chimie **58:**1069-1074.

41. **Hauser C, Kronenberg A, Allemann A, Muhlemann K, Hilty M.** 2016. Serotype/serogroup-specific antibiotic non-susceptibility of invasive and non-invasive *Streptococcus pneumoniae*, Switzerland, 2004 to 2014. Euro Surveill **21**.

42. **Oliver MB, Jones C, Larson TR, Calix JJ, Zartler ER, Yother J, Nahm MH.** 2013. *Streptococcus pneumoniae* serotype 11D has a bispecific glycosyltransferase and expresses two different capsular polysaccharide repeating units. J Biol Chem **288:**21945-21954.

43. **Yother J.** 2011. Capsules of *Streptococcus pneumoniae* and other bacteria: paradigms for polysaccharide biosynthesis and regulation. Annu Rev Microbiol **65:**563-581.

44. **Park IH, Geno KA, Yu J, Oliver MB, Kim KH, Nahm MH.** 2015. Genetic, biochemical, and serological characterization of a new pneumococcal serotype, 6H, and generation of a pneumococcal strain producing three different capsular repeat units. Clin Vaccine Immunol **22:**313-318.

45. **Camilli R, Spencer BL, Moschioni M, Pinto V, Berti F, Nahm MH, Pantosti A.** 2014. Identification of *Streptococcus pneumoniae* serotype 11E, serovariant 11Av

and mixed populations by high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and flow cytometric serotyping assay (FCSA). PLoS One **9:**e100722.

46. **Oliver MB, van der Linden MP, Kuntzel SA, Saad JS, Nahm MH.** 2013. Discovery of *Streptococcus pneumoniae* serotype 6 variants with glycosyltransferases synthesizing two differing repeating units. J Biol Chem **288:**25976-25985.

47. **Calix JJ, Porambo RJ, Brady AM, Larson TR, Yother J, Abeygunwardana C, Nahm MH.** 2012. Biochemical, genetic, and serological characterization of two capsule subtypes among *Streptococcus pneumoniae* Serotype 20 strains: discovery of a new pneumococcal serotype. J Biol Chem **287:**27885-27894.

48. **Burton RL, Geno KA, Saad JS, Nahm MH.** 2016. Pneumococcus with the "6E" cps Locus Produces Serotype 6B Capsular Polysaccharide. J Clin Microbiol **54:**967-971.

49. **Sorensen UB, Henrichsen J, Chen HC, Szu SC.** 1990. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of *Streptococcus pneumoniae* revealed by immunochemical methods. Microb Pathog **8:**325-334.

50. **Talaga P, Bellamy L, Moreau M.** 2001. Quantitative determination of C-polysaccharide in *Streptococcus pneumoniae* capsular polysaccharides by use of high-performance anion-exchange chromatography with pulsed amperometric detection. Vaccine **19:**2987-2994.

558 **TABLE 1. *Streptococcus pneumoniae* strains used in this study**

ID	Serotype	Subtype*	*plyNCR*-RFLP**	MLST
109.44	19A	19A-I	16	ST 276
501.14	19A	19A-I	1	ST 416
501.24	19A	19A-II	1	ST 199
412.49	19A	19A-III	14	ST 1151
Hungary 19A-6 (ATCC 700673)	19A	19A-III	20	ST 268***
505.32	19F	19F	4	ST 179
B201.73	19F	19F	40	ST 43

559

560 **ND: Not done; MLST: Multi Locus Sequencing Typing; ST: Sequence Type; RFLP:**

561 **Restriction Fragment Length Polymorphism**

562 *** Defined as previous described (19)**

563 **** Done as previously described (21).**

564 ***** According to: http://spneumoniae.mlst.net/sql/fulldetails.asp?id=689**

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FIGURE LEGENDS

FIGURE 1. A. Epidemiology of 19A subtypes (19A-I, 19A-II, and 19A-III) 1998-2011. A total of 158 serotype 19A strains isolated within the Swiss Sentinel Network (outpatients with upper respiratory tract infections) were analyzed. 1998-2005 was considered as pre-conjugate vaccine (PCV7) era. The % of tested isolates and subgroup assignment for both periods is shown. There was an overall significance as derived by Fisher exact test (P=0.02). Chi-Square test revealed significant changes in relative frequency between the two periods for subtypes 19A-I (p=0.02) and 19A-II (p=0.02). 95% confidence intervals (CI) are indicated. **B. Distribution of molecular types as determined by *plyNCR*-RFLP 1998-2011.** Shown are the most frequent *plyNCR*-RFLP types (Types 1 and 16). The remaining types were pooled within 'others'. There was no overall significance as derived by Fisher exact test for the two different eras. 95% confidence intervals (CI) are indicated. **C. Antibiotic resistance of serotype 19A isolates against penicillin, erythromycin, and sulfamethoxazole / trimethoprim (SXT), 1998-2011.** Minimum inhibitory concentration (MIC) for penicillin non-susceptibility was ≥ 0.06 mg/L while for erythromycin and SXT the disk diffusion method was performed (intermediate and resistant were considered as non-susceptible. 19A II is significantly more susceptible towards penicillin (Fisher exact test; p< 0.001), erythromycin (p= 0.014), and SXT (p< 0.001) as compared to the other subtypes. 95% confidence intervals (CI) are indicated.

FIGURE 2. HPLC chromatograms of serotype 19A subtypes and 19F strain B201.73 grown in CDM and PIM. HPLC composition analysis of hydrolyzed polysaccharide capsule of clinical isolates of serotype 19A subtypes grown in chemically defined medium with 55 mM glucose (A) compared to ATCC purified pneumococcal serotype 19A polysaccharide (B), and the pneumococcal inoculation medium PIM (C). Y-axis shows fluorescence (FU) and chromatograms were stacked to facilitate comparison. Peaks of mannosamine (Man-N), rhamnose (Rha) and glucose (Glc) are labeled.

595 **FIGURE 3. A. 1D NMR spectra of serotype 19A subtypes.** Shown are 1H NMR spectra of
596 capsular polysaccharide purified from each subtype grown in CDM 55 mM glucose (black)
597 and PIM (red) compared to serotype 19F and ATCC 19A purified pneumococcal
598 polysaccharide. **B. 2D NMR.** Shown is a superimposition of ^{1}H-^{13}C HSQC-NMR spectra of
599 the anomeric region from PS from Hungary 19A-6 capsule grown in CDM and PIM.